

HUMAN PLATELET F11 RECEPTOR

This application claims priority of U.S. Provisional Patent Application No. 60/100,638, filed September 16, 5 1998. The present application is a divisional of U.S. Patent Application Serial No. 09/397,243, filed September 16, 1999.

FIELD OF THE INVENTION

The subject invention is directed generally to the human platelet F11 receptor, and more particularly to 10 nucleic acid molecules encoding the human platelet F11 receptor and uses thereof.

BACKGROUND OF THE INVENTION

Throughout this application various publications are 15 referenced, many in parenthesis. Full citations for each of these publications are provided at the end of the Detailed Description. The disclosures of each of these publications in their entireties are hereby incorporated by reference in this application.

20 The significant role of platelet activation in hemostasis is well-documented. Platelet activation is necessary for platelet aggregation and secretion, and is initiated by the binding of agonist to receptors at the platelet surface. Over the last ten years, several 25 laboratories have developed monoclonal antibodies to platelet membrane glycoproteins. These platelet membrane glycoproteins can serve as agonist receptors on the platelet membrane. The antibodies to these glycoproteins have been of great value in studies designed to elucidate 30 the structure and function of these glycoproteins.

Most of the success in raising monoclonal antibodies to platelet receptors was in studies on the integrin cohesion receptor IIb/IIIa (Coller et al., 1983; Kornecki et al., 1984) and the adhesion receptor Ib/IX (Coller et 35 al., 1983; Handa et al., 1986). Functional antibodies that inhibit the action of these receptors provided a

large body of new information and have led to direct conclusions about the functions of these glycoprotein receptors. Such inhibitory antibodies were also shown to have potential *in vivo* therapeutic use (Coller et al., 5 1986; Peters et al., 1986).

Some of these antibodies that serve as agonists, binding to the receptors at the platelet surface and thereby activating the platelets, have been identified. Several laboratories have developed or identified such 10 "activator" antibodies that appear to react with platelet membrane protein components of 21-24 kD. The first report of a monoclonal antibody which served as an agonist and induced platelet aggregation was published by Boucheix et al. in 1983. This monoclonal antibody 15 immunoprecipitated a platelet protein with apparent molecular weight (M.W.) of 24 kD under both reduced and non-reduced conditions. The addition of Fab fragments of this antibody to platelets resulted in the inhibition of platelet aggregation induced by various agonists. The 20 platelet antigen recognized by this antibody was identical to the leukemia-associated antigen, p24, found in common acute lymphoblastic leukemia cells and neuroblastoma cells (Kersey et al., 1981; Jones et al., 1982; Komada et al., 1983).
25 Thiagarajan et al. (1983) reported that platelet aggregation could be induced by another monoclonal antibody. This antibody was found to be directed against a 21 kD protein present in both normal and Glanzmann's thrombasthenic platelets.
30 Gorman et al. (1985) have described several monoclonal antibodies which induce platelet aggregation. All of these antibodies immunoprecipitated a 24 kD platelet protein in both the reduced and non-reduced states. The Fab fragments of these antibodies were found

to augment the aggregation of platelets by adenosine diphosphate (ADP).

Higashihara et al. (1985) also described a monoclonal antibody which induced platelet aggregation and secretion by interaction with a protein of 24 kD. Preincubation of platelets with this antibody inhibited ristocetin-induced agglutination. It is known that these antibodies are directed against the p24/CD9 protein on the platelet surface. The CD9 antigen has been cloned and sequenced (Boucheix et al., 1991; Lanza et al., 1991), and CD9 antibodies have been shown to induce platelet aggregation mediated by the Fc γ RII receptor (CD32 molecule) (Worthington et al., 1990).

Duncan and Rosse (1986) showed that antibodies to platelet HLA class I antigen (anti-ABH IgG) could activate platelets and induce serotonin release. Similar results were obtained by Cosgrove et al. (1988), who reported that three different anti-HLA Class I monoclonal antibodies and an anti- β_2 microglobulin antibody caused platelet aggregation and secretion. Duncan and Rosse (1986) also showed that high concentrations of anti-PL A^1 antibodies inhibited platelet secretion induced by these antibodies. Ryu et al. (1989) found that high concentrations of PL A^1 blocked fibrinogen binding resulting in the blockage of agonist-induced platelet aggregation, whereas low concentrations of anti-PL A^1 antibodies induced release and aggregation.

Activator monoclonal antibodies directed against GPIIb and GPIIIa have also been reported. A stimulatory monoclonal antibody to the GPIIb/IIIa complex has been described by Modderman et al. (1988) which induces the release of alpha and dense granule contents resulting in platelet aggregation. Morel et al. (1989) have described a monoclonal antibody directed against GPIIb. The F(ab') $_2$

fragments of this antibody did not induce platelet aggregation although they blocked the stimulation of platelets by the intact antibody.

In addition to these antibodies, antibodies of other specificity have been described which activate platelets. Scott et al. (1989) described a monoclonal antibody which stimulates platelet secretion and aggregation and is directed against a platelet membrane glycoprotein of M.W. 67 kD. Recently, Yanabu et al. (1991) detected an autoantibody in a patient with immunothrombocytopenia (ITP), which activated normal platelets by interacting with a 36 kD platelet surface protein.

Kornecki et al. (1990) referred to a monoclonal antibody called M.Ab.F11 which induces vesicular secretion and aggregation in human platelets. U.S. Patent No. 5,665,701, issued September 9, 1997, of Kornecki et al. (the entire contents of which are incorporated herein by reference) discloses further details of the F11 receptor protein, including partial amino acid sequences. Although the Patent refers to DNA encoding the platelet membrane glycoprotein F11 (column 4, line 10), no nucleotide sequences encoding the protein are provided or described.

The health related significance of these antibodies which can activate human platelets is apparent. Characterization of the antigens which serve as receptors for these antibodies in the activation process is necessary as well as the elucidation of the biochemical pathways triggered by these interactions.

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SUMMARY OF THE INVENTION

To this end, the subject invention provides an isolated nucleic acid molecule encoding a human platelet F11 receptor. The invention also provides an antisense

nucleic acid molecule complementary to at least a portion of the mRNA encoding the human platelet F11 receptor.

The isolated nucleic acid molecules of the invention can be inserted into suitable expression vectors and/or host cells. Expression of the nucleic acid molecules encoding the human platelet F11 receptor results in production of human platelet F11 receptor in a host cell. Expression of the antisense nucleic acid molecules in a host cell results in decreased expression of the human platelet F11 receptor.

The invention further provides a ribozyme having a recognition sequence complementary to a portion of mRNA encoding a human platelet F11 receptor. The ribozyme can be introduced into a cell to also achieve decreased expression of human platelet F11 receptor in the cell.

The invention further provides a method of screening a substance for the ability of the substance to modify F11 receptor function, and a method of obtaining DNA encoding a human platelet F11 receptor.

Further provided is an isolated nucleic acid molecule encoding a human platelet F11 receptor, wherein the nucleic acid molecule encodes a first amino acid sequence having at least 90% amino acid identity to a second amino acid sequence. The second amino acid sequence has an amino acid sequence selected from the group consisting of SEQ ID NO:3, amino acid residues 28-299 of SEQ ID NO:3, SEQ ID NO:4, and amino acid residues 28-193 of SEQ ID NO:4.

The invention further provides a DNA oligomer capable of hybridizing to a nucleic acid molecule encoding a human platelet F11 receptor. The DNA oligomer can be used in a method of detecting presence of a human platelet F11 receptor in a sample, which method is also provided by the subject invention.

The subject invention is based on the discovery that a stimulatory monoclonal antibody (termed M.Ab.F11) induces aggregation and granule secretion of human platelets through its specific binding to a platelet membrane glycoprotein duplex of MW 32 and 35 kDa, termed F11 receptor (also referred to as FAM or F11-Adhesion Molecule). Internal amino acid sequences of FAM enabled the cloning of a full-length cDNA. The predicted amino acid sequence revealed that FAM is an integral membrane protein. An extracellular N-terminal region consisting of two Ig-like C2-type domains is followed by a transmembrane domain and a short cytoplasmic tail. FAM is thus a new member of the Ig gene superfamily. FAM showed 69% homology with the murine junctional adhesion molecule (JAM). An immunocytochemical location in the tight junctions of HUVEC cells, similar to that shown by JAM in mouse cells, suggests a role for FAM in cell-cell adhesion in addition to participation in platelet activation. By Western blotting, a number of myeloid and vascular cells were found to express FAM. The detection of autoantibodies against FAM in the circulation of thrombocytopenic and renal patients indicates that FAM may play a pathophysiological role in clinical disorders involving the activation of platelets *in vivo*.

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BRIEF DESCRIPTION OF THE DRAWINGS

These and other features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawings in which:

Fig. 1 illustrates the detection of FAM in human platelets by use of a polyclonal antibody directed against the twenty-one amino acid sequence of the N-terminus of purified FAM. Total platelet proteins,

solubilized from approximately 2×10^7 platelets, were resolved on a 15% SDS-polyacrylamide gel. For immunoblotting, an anti-FAM polyclonal antibody, developed against the N-terminal sequence, was used at a 5 concentration of 20 mg/ml. The arrows point to the 32/35kDa duplex;

Fig. 2 illustrates the human platelet FAM nucleotide sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:3). The N-terminal amino acid sequence of the 10 mature FAM (SEQ ID NO:5: SVTVHSSEPEVRIPENNPVKLS) and internal sequences of peptides GluC-(4) (SEQ ID NO:6: WKFDQGDTTRLVCYNNKITASYEDRVTFLPTGITFKSVTRED) and GluC-(2) (SEQ ID NO:7: KVKLIVLV) are underlined. The start codon is indicated by a double line;

15 Fig. 3 is a hydrophobicity plot of amino acid sequences of FAM. The transmembrane domain was found in the region spanning amino acids 239 to 258 of SEQ ID NO:3, and the signal peptide sequence is found at amino acids 12 to 29 of SEQ ID NO:3;

20 Figs. 4A and 4B illustrate that FAM is a member of the Ig family. The FAM molecule contains two C2-type Ig domains. These figures show the alignment of the C2-type Ig domain consensus sequence profiles with FAM at amino acid 41-116 of SEQ ID NO:3 (Fig. 4A, SEQ ID NO:9) and 25 with FAM at amino acids 144-219 of SEQ ID NO:3 (Fig. 4B, SEQ ID NO:11). The conserved cysteine residues are boxed;

Fig. 5 is a figure of a schematic model illustrating the main deduced structural features of FAM. TM indicates the transmembrane region of FAM (shaded 30 region);

Fig. 6 illustrates the alignment of the amino acid sequences of the FAM with the Junctional Adhesion Molecule (JAM) (Bachelot et al. 1990) and the A33 molecule (Jennings et al. 1990). The C-2/V Ig fold for

each protein are indicated by the dashed lines. TM = transmembrane region;

Figs. 7A-7H illustrate the cellular distribution of FAM as determined by flow cytometry. Each cell type was 5 incubated with M.Ab.F11 (5mg/ml), followed by the addition of the secondary antibody GAM IgG-FITC. Cells were analyzed by FAC Sort flow cytometry. As controls, cells were incubated with nonimmune mouse IgG, followed by incubation with the secondary antibody. Cells 10 considered positive for FAM exhibited a mean fluorescence intensity of 40 channels higher than control groups. Each figure is representative of 5 experiments. Fig. 7A = platelets; Fig. 7B = CMK; Fig. 7C = HEL; Fig. 7D = K562; Fig. 7E = erythrocytes; Fig. 7F = lymphocytes; Fig. 7G = 15 HUVEC; Fig. 7H = Eahy 926;

Fig. 7I illustrates the cellular distribution of FAM as determined by immunoblotting. Expression of the FAM in platelets (lane 1), endothelial cells HUVEC (lane 2), HEL cells (lane 3), human erythrocytes (lane 4), 20 endothelial BAEC (lane 5) and CMK 11-5 cells (lane 6) is shown. Proteins were separated on 5-15% polyacrylamide gel (lanes 1-4) or by use of 12% SDS-PAGE (lanes 5-6). Immunoblotting was performed using M.Ab.F11 (4 µg/ml);

Fig. 8 illustrates the identification of FAM 25 antibodies in the circulation of thrombocytopenic and renal disorder patients. FAM (5mg applied/lane), was purified from platelet plasma membranes using the affinity chromatographic procedures previously described (Kornecki et al. 1990; Naik et al. 1995) and resolved by 30 SDS-PAGE. Immunoblotting was performed using the following antibodies: Lane 1, incubation with monoclonal antibody M.Ab.F11 (4 mg/ml); Lane 2, control, incubation with only 2° antibody, rabbit anti-mouse Ig; Lane 3, incubation with purified Ig fraction obtained from the

serum of a thrombocytopenic patient (25 mg/ml); Lane 4, incubation with plasma obtained from a renal disorder patient, Lanes 5 and 6, incubation with plasmas obtained from two separate healthy donors and used for 5 immunoblotting, Lane 7, control using 2° antibody, rabbit anti-human Ig.

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations Used Herein

10 ACD (acid citrate dextrose)
BAEC (bovine aortic endothelial cells)
DMEM (Dulbecco's modified Eagle's medium)
F11R (F11 receptor)
GP (glycoprotein)

15 HAT (hypoxanthine-aminopterin-thymidine)
HBS (HEPES-buffered saline)
HEL (human erythroleukemic cell line)
HMM (Hidden Markov Model)
HUVEC (human umbilical vein endothelial cells)

20 ICAM (intercellular adhesion molecule)
IgSF (Immunoglobulin superfamily)
M.Ab. (monoclonal antibody)
MadCAM-1 (mucosal addressin cell adhesion molecule-1)
NCAM (neural cell adhesion molecule)

25 PDGF (platelet-derived growth factor)
PECAM-1 (platelet endothelial cell adhesion molecule-1)
PGE₁ (prostaglandin E₁)
RACE (Rapid Amplification of cDNA Ends)
RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction)

30 SMART (Simple Modular Architecture Research Tool)
VCAM-1 (vascular cell adhesion molecule-1)

Monoclonal antibody M.Ab.F11 (directed to the F11 receptor) has been deposited pursuant to, and in

satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the American Type Culture Collection (ATCC), 10801

5 University Blvd., Manassas, VA 20110-2209 under ATCC
Accession No. HB-11761 on November 10, 1994.

The term "nucleic acid", as used herein, refers to either DNA or RNA. "Nucleic acid sequence" or "polynucleotide sequence" refers to a single- or 10 double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes both self-replicating plasmids, infectious polymers of DNA or RNA, and nonfunctional DNA or RNA.

"Isolated" nucleic acid refers to nucleic acid which 15 has been separated from an organism in a substantially purified form (i.e. substantially free of other substances originating from that organism), and to synthetic nucleic acid.

By a nucleic acid sequence "homologous to" or 20 "complementary to", it is meant a nucleic acid that selectively hybridizes, duplexes or binds to DNA sequences encoding the F11 receptor (protein) or portions thereof when the DNA sequences encoding the protein are present in a human genomic or cDNA library. A DNA 25 sequence which is similar or complementary to a target sequence can include sequences which are shorter or longer than the target sequence so long as they meet the functional test set forth.

Typically, the hybridization is done in a Southern 30 blot protocol using a 0.2X SSC, 0.1% SDS, 65°C wash. The term "SSC" refers to a citrate-saline solution of 0.15M sodium chloride and 20 mM sodium citrate. Solutions are often expressed as multiples or fractions of this concentration. For example, 6X SSC refers to a solution

having a sodium chloride and sodium citrate concentration of 6 times this amount or 0.9 M sodium chloride and 120 mM sodium citrate. 0.2X SSC refers to a solution 0.2 times the SSC concentration or 0.03M sodium chloride and 5 4 mM sodium citrate.

The phrase "nucleic acid molecule encoding" refers to a nucleic acid molecule which directs the expression of a specific protein or peptide. The nucleic acid sequences include both the DNA strand sequence that is 10 transcribed into RNA and the RNA sequence that is translated into protein or peptide. The nucleic acid molecule includes both the full length nucleic acid sequences as well as non-full length sequences derived from the full length protein. It being further 15 understood that the sequence includes the degenerate codons of the native sequence or sequences which may be introduced to provide codon preference in a specific host cell.

The term "located upstream" as used herein refers to 20 linkage of a promoter upstream from a nucleic acid (DNA) sequence such that the promoter mediates transcription of the nucleic acid (DNA) sequence.

The term "vector", refers to viral expression systems, autonomous self-replicating circular DNA 25 (plasmids), and includes both expression and nonexpression plasmids. Where a recombinant microorganism or cell is described as hosting an "expression vector," this includes both extrachromosomal circular DNA and DNA that has been incorporated into the 30 host chromosome(s). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous structure, or the vector may be incorporated within the host's genome.

The term "plasmid" refers to an autonomous circular DNA molecule capable of replication in a cell, and includes both the expression and nonexpression types. Where a recombinant microorganism or cell is described as 5 hosting an "expression plasmid", this includes latent viral DNA integrated into the host chromosome(s). Where a plasmid is being maintained by a host cell, the plasmid is either being stably replicated by the cell during mitosis as an autonomous structure, or the plasmid is 10 incorporated within the host's genome.

The phrase "heterologous protein" or "recombinantly produced heterologous protein" refers to a peptide or protein of interest produced using cells that do not have an endogenous copy of DNA able to express the peptide or 15 protein of interest. The cells produce the peptide or protein because they have been genetically altered by the introduction of the appropriate nucleic acid sequences. The recombinant peptide or protein will not be found in association with peptides or proteins and other 20 subcellular components normally associated with the cells producing the peptide or protein.

The following terms are used to describe the sequence relationships between two or more nucleic acid molecules or polynucleotides, or between two or more 25 amino acid sequences of peptides or proteins: "reference sequence", "comparison window", "sequence identity", "sequence homology", "percentage of sequence identity", "percentage of sequence homology", "substantial identity", and "substantial homology". A "reference 30 sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing or may comprise a complete cDNA or gene sequence.

Optimal alignment of sequences for aligning a comparison window may be conducted, for example, by the local homology algorithm of Smith and Waterman (1981), by the homology alignment algorithm of Needleman and Wunsch 5 (1970), by the search for similarity method of Pearson and Lipman (1988), or by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.).

10 As applied to nucleic acid molecules or polynucleotides, the terms "substantial identity" or "substantial sequence identity" mean that two nucleic acid sequences, when optimally aligned (see above), share at least 90 percent sequence identity, preferably at 15 least 95 percent sequence identity, more preferably at least 96, 97, 98 or 99 percent sequence identity.

"Percentage nucleotide (or nucleic acid) identity" or "percentage nucleotide (or nucleic acid) sequence identity" refers to a comparison of the nucleotides of 20 two nucleic acid molecules which, when optimally aligned, have approximately the designated percentage of the same nucleotides. For example, "95% nucleotide identity" refers to a comparison of the nucleotides of two nucleic acid molecules which when optimally aligned have 95% 25 nucleotide identity. Preferably, nucleotide positions which are not identical differ by redundant nucleotide substitutions (the nucleotide substitution does not change the amino acid encoded by the particular codon).

As further applied to nucleic acid molecules or 30 polynucleotides, the terms "substantial homology" or "substantial sequence homology" mean that two nucleic acid sequences, when optimally aligned (see above), share at least 90 percent sequence homology, preferably at

least 95 percent sequence homology, more preferably at least 96, 97, 98 or 99 percent sequence homology.

"Percentage nucleotide (or nucleic acid) homology" or "percentage nucleotide (or nucleic acid) sequence homology" refers to a comparison of the nucleotides of two nucleic acid molecules which, when optimally aligned, have approximately the designated percentage of the same nucleotides or nucleotides which are not identical but differ by redundant nucleotide substitutions (the 10 nucleotide substitution does not change the amino acid encoded by the particular codon). For example, "95% nucleotide homology" refers to a comparison of the nucleotides of two nucleic acid molecules which when optimally aligned have 95% nucleotide homology.

15 As applied to polypeptides, the terms "substantial identity" or "substantial sequence identity" mean that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap, share at least 90 percent sequence identity, preferably at least 20 95 percent sequence identity, more preferably at least 96, 97, 98 or 99 percent sequence identity.

"Percentage amino acid identity" or "percentage amino acid sequence identity" refers to a comparison of the amino acids of two polypeptides which, when optimally aligned, have approximately the designated percentage of the same amino acids. For example, "95% amino acid identity" refers to a comparison of the amino acids of two polypeptides which when optimally aligned have 25 95% amino acid identity. Preferably, residue positions which 30 are not identical differ by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to affect the properties of a

protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.

As further applied to polypeptides, the terms "substantial homology" or "substantial sequence homology" mean that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap, share at least 90 percent sequence homology, preferably at least 95 percent sequence homology, more preferably at least 96, 97, 98 or 99 percent sequence homology.

"Percentage amino acid homology" or "percentage amino acid sequence homology" refers to a comparison of the amino acids of two polypeptides which, when optimally aligned, have approximately the designated percentage of the same amino acids or conservatively substituted amino acids. For example, "95% amino acid homology" refers to a comparison of the amino acids of two polypeptides which when optimally aligned have 95% amino acid homology. As used herein, homology refers to identical amino acids or residue positions which are not identical but differ only by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to affect the properties of a protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.

The phrase "substantially purified" or "isolated" when referring to a protein (or peptide), means a chemical composition which is essentially free of other cellular components. It is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein (or peptide) which is

the predominant species present in a preparation is substantially purified. Generally, a substantially purified or isolated protein (or peptide) will comprise more than 80% of all macromolecular species present in 5 the preparation. Preferably, the protein (or peptide) is purified to represent greater than 90% of all macromolecular species present. More preferably the protein (or peptide) is purified to greater than 95%, and most preferably the protein (or peptide) is purified to 10 essential homogeneity, wherein other macromolecular species are not detected by conventional techniques. A "substantially purified" or "isolated" protein (or peptide) can be separated from an organism, synthetically or chemically produced, or recombinantly produced.

15 "Biological sample" or "sample" as used herein refers to any sample obtained from a living organism or from an organism that has died. Examples of biological samples include body fluids and tissue specimens.

High stringent hybridization conditions are selected 20 to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, 25 stringent conditions will be those in which the salt concentration is at least about 0.02 molar at pH 7 and the temperature is at least about 60°C. As other factors may significantly affect the stringency of hybridization, including, among others, base composition and size of the 30 complementary strands, the presence of organic solvents, ie. salt or formamide concentration, and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one. High stringency may be attained, for example, by overnight

hybridization at about 68°C in a 6X SSC solution, washing at room temperature with 6X SSC solution, followed by washing at about 68°C in a 6X SSC solution then in a 0.6X SSX solution.

5 Hybridization with moderate stringency may be attained, for example, by: 1) filter pre-hybridizing and hybridizing with a solution of 3X sodium chloride, sodium citrate (SSC), 50% formamide, 0.1M Tris buffer at pH 7.5, 5X Denhardt's solution; 2) pre-hybridization at 37°C for
10 4 hours; 3) hybridization at 37°C with amount of labelled probe equal to 3,000,000 cpm total for 16 hours; 4) wash in 2X SSC and 0.1% SDS solution; 5) wash 4X for 1 minute each at room temperature and 4X at 60°C for 30 minutes each; and 6) dry and expose to film.

15 The phrase "selectively hybridizing to" refers to a nucleic acid molecule that hybridizes, duplexes or binds only to a particular target DNA or RNA sequence when the target sequences are present in a preparation of total cellular DNA or RNA. By selectively hybridizing it is
20 meant that a nucleic acid molecule binds to a given target in a manner that is detectable in a different manner from non-target sequence under moderate, or more preferably under high, stringency conditions of hybridization. "Complementary" or "target" nucleic acid
25 sequences refer to those nucleic acid sequences which selectively hybridize to a nucleic acid molecule. Proper annealing conditions depend, for example, upon a nucleic acid molecule's length, base composition, and the number of mismatches and their position on the molecule, and
30 must often be determined empirically. For discussions of nucleic acid molecule (probe) design and annealing conditions, see, for example, Sambrook et al. 1989.

It will be readily understood by those skilled in the art and it is intended here, that when reference is

made to particular sequence listings, such reference includes sequences which substantially correspond to its complementary sequence and those described including allowances for minor sequencing errors, single base 5 changes, deletions, substitutions and the like, such that any such sequence variation corresponds to the nucleic acid sequence of the peptide/protein to which the relevant sequence listing relates.

The DNA molecules of the subject invention also 10 include DNA molecules coding for protein (receptor) analogs, fragments or derivatives of the protein which differ from naturally-occurring forms (the naturally- occurring protein) in terms of the identity or location of one or more amino acid residues (deletion analogs 15 containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues, and addition analogs wherein one or more amino acid residues is added to a terminal or medial portion of the protein) 20 and which share the function of the naturally-occurring form. These molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of 25 additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

As used herein, a "peptide" refers to an amino acid sequence of three to one hundred amino acids, and 30 therefore an isolated peptide that comprises an amino acid sequence is not intended to cover amino acid sequences of greater than 100 amino acids. Preferably, peptides are less than 50 amino acids in length, and more

preferably the peptides are five to 20 amino acids in length or 20-40 amino acids in length.

Peptides or proteins can contain any naturally-occurring or non-naturally-occurring amino acids, including the D-form of the amino acids, amino acid derivatives and amino acid mimics, so long as the desired function and activity of the peptide or protein is maintained. The choice of including an (L)- or a (D)-amino acid in the peptides or proteins depends, in part, on the desired characteristics of the peptide or protein. For example, the incorporation of one or more (D)-amino acids can confer increased stability on the peptide or protein and can allow a peptide or protein to remain active in the body for an extended period of time. The incorporation of one or more (D)-amino acids can also increase or decrease the pharmacological activity of the peptide or protein.

The peptides or proteins may also be cyclized, since cyclization may provide the peptides or proteins with superior properties over their linear counterparts.

As used herein, the terms "amino acid mimic" and "mimetic" mean an amino acid analog or non-amino acid moiety that has the same or similar functional characteristic of a given amino acid. For instance, an amino acid mimic of a hydrophobic amino acid is one which is non-polar and retains hydrophobicity, generally by way of containing an aliphatic chemical group. By way of further example, an arginine mimic can be an analog of arginine which contains a side chain having a positive charge at physiological pH, as is characteristic of the guanidinium side chain reactive group of arginine.

In addition, modifications to the peptide backbone and peptide bonds thereof are also encompassed within the scope of amino acid mimic or mimetic. Such modifications

can be made to the amino acid, derivative thereof, non-amino acid moiety or the peptide either before or after the amino acid, derivative thereof or non-amino acid moiety is incorporated into the peptide. What is critical is that such modifications mimic the peptide backbone and bonds which make up the same and have substantially the same spacial arrangement and distance as is typical for traditional peptide bonds and backbones. An example of one such modification is the reduction of the carbonyl(s) of the amide peptide backbone to an amine. A number of reagents are available and well known for the reduction of amides to amines such as those disclosed in Wann et al. (1981) and Raucher et al. (1980). An amino acid mimic is, therefor, an organic molecule that retains the similar amino acid pharmacophore groups as is present in the corresponding amino acid and which exhibits substantially the same spatial arrangement between functional groups.

The substitution of amino acids by non-naturally occurring amino acids and amino acid mimics as described above can enhance the overall activity or properties of an individual peptide or protein based on the modifications to the backbone or side chain functionalities. For example, these types of alterations to the specifically described amino acid substituents and exemplified peptides or proteins can enhance the peptide or protein's stability to enzymatic breakdown and increase biological activity. Modifications to the peptide backbone similarly can add stability and enhance activity.

With these definitions in mind, the subject invention provides an isolated nucleic acid molecule encoding a human platelet F11 receptor. The nucleic acid molecule can be deoxyribonucleic acid (DNA) or

ribonucleic acid (RNA, including messenger RNA or mRNA), genomic or recombinant, biologically isolated or synthetic.

The DNA molecule can be a cDNA molecule, which is a
5 DNA copy of a messenger RNA (mRNA) encoding the F11 receptor.

An example of a nucleic acid molecule which encodes a human platelet F11 receptor is the molecule having the nucleotide sequence as shown in SEQ ID NO:1. Nucleotides
10 16-96 of SEQ ID NO:1 represent a signal sequence, and nucleotides 97-912 represent the mature protein (nucleotides 16-912 thus represent the F11 receptor with its signal sequence). Nucleotides 16-18 are an ATG start codon, and nucleotides 913-915 are a stop codon. The
15 amino acid sequence encoded by this nucleotide sequence is shown in SEQ ID NO:3. Amino acid residues 1-27 of SEQ ID NO:3 represent the signal sequence, and amino acid residues 28-299 represent the mature protein (amino acid residues 1-299 thus represent the F11 receptor with its
20 signal sequence). This F11 receptor is designated F11R-A. Another example of a nucleic acid molecule which encodes a human platelet F11 receptor is the molecule having the nucleotide sequence as shown in SEQ ID NO:2. Nucleotides 16-96 of SEQ ID NO:2 represent a signal
25 sequence, and nucleotides 97-594 represent the mature protein (nucleotides 16-594 thus represent the F11 receptor with its signal sequence). Nucleotides 16-18 are an ATG start codon, and nucleotides 595-597 are a stop codon. The amino acid sequence encoded by this
30 nucleotide sequence is shown in SEQ ID NO:4. Amino acid residues 1-27 of SEQ ID NO:4 represent the signal sequence, and amino acid residues 28-193 represent the mature protein (amino acid residues 1-193 thus represent

the F11 receptor with its signal sequence). This F11 receptor is designated F11R-B.

The invention also provides an antisense nucleic acid molecule that is complementary to at least a portion 5 of the mRNA encoding the human platelet F11 receptor. Antisense nucleic acid molecules can be RNA or single-stranded DNA, and can be complementary to the entire mRNA molecule encoding the F11 receptor (i.e. of the same nucleotide length as the entire molecule). It may be 10 desirable, however, to work with a shorter molecule. In this instance, the antisense molecule can be complementary to a portion of the entire mRNA molecule encoding the F11 receptor. These shorter antisense molecules are capable of hybridizing to the mRNA encoding 15 the entire molecule, and preferably consist of about twenty to about one hundred nucleotides. These antisense molecules can be used to reduce levels of human platelet F11 receptor, by introducing into cells an RNA or single-stranded DNA molecule that is complementary to at least a 20 portion of the mRNA of the F11 receptor (i.e. by introducing an antisense molecule). The antisense molecule can base-pair with the mRNA of the F11 receptor, preventing translation of the mRNA into protein. Thus, 25 an antisense molecule to the F11 receptor can prevent translation of mRNA encoding the F11 receptor into a functional F11 receptor protein.

More particularly, an antisense molecule complementary to at least a portion of mRNA encoding a human platelet F11 receptor can be used to decrease 30 expression of a functional F11 receptor. A cell with a first level of expression of a functional human platelet F11 receptor is selected, and then the antisense molecule is introduced into the cell. The antisense molecule blocks expression of functional human platelet F11

receptor, resulting in a second level of expression of a functional human platelet F11 receptor in the cell. The second level is less than the initial first level.

Antisense molecules can be introduced into cells by 5 any suitable means. In one embodiment, the antisense RNA molecule is injected directly into the cellular cytoplasm, where the RNA interferes with translation. A vector may also be used for introduction of the antisense molecule into a cell. Such vectors include various 10 plasmid and viral vectors. For a general discussion of antisense molecules and their use, see Han et al. 1991 and Rossi 1995.

The invention further provides a special category of antisense RNA molecules, known as ribozymes, having 15 recognition sequences complementary to specific regions of the mRNA encoding the human platelet F11 receptor. Ribozymes not only complex with target sequences via complementary antisense sequences but also catalyze the hydrolysis, or cleavage, of the template mRNA molecule. 20 Examples, which are not intended to be limiting, of suitable regions of the mRNA template to be targeted by ribozymes are any of the homologous regions identified by comparing the various F11 receptors.

Expression of a ribozyme in a cell can inhibit gene 25 expression (such as the expression of a human platelet F11 receptor). More particularly, a ribozyme having a recognition sequence complementary to a region of a mRNA encoding a human platelet F11 receptor can be used to decrease expression of human platelet F11 receptor. A 30 cell with a first level of expression of human platelet F11 receptor is selected, and then the ribozyme is introduced into the cell. The ribozyme in the cell decreases expression of human platelet F11 receptor in

the cell, because mRNA encoding the human platelet F11 receptor is cleaved and cannot be translated.

Ribozymes can be introduced into cells by any suitable means. In one embodiment, the ribozyme is 5 injected directly into the cellular cytoplasm, where the ribozyme cleaves the mRNA and thereby interferes with translation. A vector may be used for introduction of the ribozyme into a cell. Such vectors include various plasmid and viral vectors (note that the DNA encoding the 10 ribozyme does not need to be "incorporated" into the genome of the host cell; it could be expressed in a host cell infected by a viral vector, with the vector expressing the ribozyme, for instance). For a general discussion of ribozymes and their use, see Sarver et al. 15 1990, Chrisey et al. 1991, Rossi et al. 1992, and Christoffersen et al. 1995.

The nucleic acid molecules of the subject invention can be expressed in suitable host cells using conventional techniques. Any suitable host and/or vector 20 system can be used to express the human platelet F11 receptor. For in vitro expression, bacterial hosts (for example, *Escherichia coli*) and mammalian hosts (for example, Hela cells, Cv-1 cells, COS cells) are preferred. For in vivo expression, the most suitable 25 host cell depends on the goal of the expression.

Techniques for introducing the nucleic acid molecules into the host cells may involve the use of expression vectors which comprise the nucleic acid molecules. These expression vectors (such as plasmids 30 and viruses; viruses including bacteriophage) can then be used to introduce the nucleic acid molecules into suitable host cells. For example, DNA encoding the human platelet F11 receptor can be injected into the nucleus of a host cell or transformed into the host cell using a

suitable vector, or mRNA encoding the human platelet F11 receptor can be injected directly into the host cell, in order to obtain expression of the human platelet F11 receptor in the host cell.

- 5 Various methods are known in the art for introducing nucleic acid molecules into host cells. One method is microinjection, in which DNA is injected directly into the nucleus of cells through fine glass needles (or RNA is injected directly into the cytoplasm of cells).
- 10 Alternatively, DNA can be incubated with an inert carbohydrate polymer (dextran) to which a positively charged chemical group (DEAE, for diethylaminoethyl) has been coupled. The DNA sticks to the DEAE-dextran via its negatively charged phosphate groups. These large DNA-
- 15 containing particles stick in turn to the surfaces of cells, which are thought to take them in by a process known as endocytosis. Some of the DNA evades destruction in the cytoplasm of the cell and escapes to the nucleus, where it can be transcribed into RNA like any other gene
- 20 in the cell. In another method, cells efficiently take in DNA in the form of a precipitate with calcium phosphate. In electroporation, cells are placed in a solution containing DNA and subjected to a brief electrical pulse that causes holes to open transiently in
- 25 their membranes. DNA enters through the holes directly into the cytoplasm, bypassing the endocytotic vesicles through which they pass in the DEAE-dextran and calcium phosphate procedures. DNA can also be incorporated into artificial lipid vesicles, liposomes, which fuse with the
- 30 cell membrane, delivering their contents directly into the cytoplasm. In an even more direct approach, DNA is absorbed to the surface of tungsten microprojectiles and fired into cells with a device resembling a shotgun.

Several of these methods, microinjection, electroporation, and liposome fusion, have been adapted to introduce proteins into cells. For review, see Mannino and Gould-Fogerite 1988, Shigekawa and Dower 5 1988, Capecchi 1980, and Klein et al. 1987.

Further methods for introducing nucleic acid molecules into cells involve the use of viral vectors. One such virus widely used for protein production is an insect virus, baculovirus. For a review of baculovirus 10 vectors, see Miller (1989). Various viral vectors have also been used to transform mammalian cells, such as bacteriophage, vaccinia virus, adenovirus, and retrovirus.

As indicated, some of these methods of transforming 15 a cell require the use of an intermediate plasmid vector. U.S. Patent No. 4,237,224 to Cohen and Boyer describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids 20 are then introduced by means of transformation and replicated in unicellular cultures including prokaryotic organisms and eucaryotic cells grown in tissue culture. The DNA sequences are cloned into the plasmid vector using standard cloning procedures known in the art, as 25 described by Sambrook et al. (1989).

Host cells into which the nucleic acid encoding the human platelet F11 receptor has been introduced can be used to produce (i.e. to functionally express) the human platelet F11 receptor. The function of the encoded human 30 platelet F11 receptor can be assayed according to methods known in the art.

Having identified the nucleic acid molecules encoding human platelet F11 receptors and methods for expressing the human platelet F11 receptors encoded

thereby, the invention further provides a method of screening a substance (for example, a compound or inhibitor) for the ability of the substance to modify F11 receptor function. The method comprises introducing a 5 nucleic acid molecule encoding the human platelet F11 receptor into a host cell, and expressing the human platelet F11 receptor encoded by the molecule in the host cell. The cell is then exposed to a substance and evaluated to determine if the substance modifies the 10 function of the F11 receptor. From this evaluation, substances effective in altering the function of the F11 receptor can be found. Such agents may be, for example, small molecule inhibitors, chemicals, peptides, antibodies, etc.

15 The evaluation of the cell to determine if the substance modifies the function of the F11 receptor can be by any means known in the art.

The nucleic acid molecules of the subject invention can be used either as probes or for the design of primers 20 to obtain DNA encoding other human platelet F11 receptors by either cloning and colony/plaque hybridization or amplification using the polymerase chain reaction (PCR).

Specific probes derived from SEQ ID NO:1 or SEQ ID NO:2 (or portions thereof) can be employed to identify 25 colonies or plaques containing cloned DNA encoding a member of the human platelet F11 receptor family using known methods (see Sambrook et al. 1989). One skilled in the art will recognize that by employing such probes under high stringency conditions (for example, 30 hybridization at 42°C with 5X SSPC and 50% formamide, washing at 50-65°C with 0.5X SSPC), sequences having regions which are greater than 90% homologous or identical to the probe can be obtained. Sequences with lower percent homology or identity to the probe, which

also encode human platelet F11 receptors, can be obtained by lowering the stringency of hybridization and washing (e.g., by reducing the hybridization and wash temperatures or reducing the amount of formamide employed).

More particularly, in one embodiment, the method comprises selection of a DNA molecule encoding a human platelet F11 receptor, or a fragment thereof, the DNA molecule having a nucleotide sequence as shown in SEQ ID NO:1, nucleotides 16-912 of SEQ ID NO:1, nucleotides 97-912 of SEQ ID NO:1, SEQ ID NO:2, nucleotides 16-594 of SEQ ID NO:2, or nucleotides 97-594 of SEQ ID NO:2, and designing an oligonucleotide probe for human platelet F11 receptor based on the nucleotide sequence of the selected DNA molecule. A genomic or cDNA library of an organism is then probed with the oligonucleotide probe, and clones are obtained from the library that are recognized by the oligonucleotide probe so as to obtain DNA encoding another human platelet F11 receptor.

Specific primers derived from SEQ ID NO:1 or SEQ ID NO:2 (or portions thereof) can be used in PCR to amplify a DNA sequence encoding a member of the human platelet F11 receptor family using known methods (see Innis et al. 1990). One skilled in the art will recognize that by employing such primers under high stringency conditions (for example, annealing at 50-60°C, depending on the length and specific nucleotide content of the primers employed), sequences having regions greater than 75% homologous or identical to the primers will be amplified.

More particularly, in a further embodiment the method comprises selection of a DNA molecule encoding human platelet F11 receptor, or a fragment thereof, the DNA molecule having a nucleotide sequence as shown in SEQ ID NO:1, nucleotides 16-912 of SEQ ID NO:1, nucleotides

97-912 of SEQ ID NO:1, SEQ ID NO:2, nucleotides 16-594 of SEQ ID NO:2, or nucleotides 97-594 of SEQ ID NO:2, designing degenerate oligonucleotide primers based on the nucleotide sequence of the selected DNA molecule, and
5 employing such primers in the polymerase chain reaction using as a template a DNA sample to be screened for the presence of human platelet F11 receptor-encoding sequences. The resulting PCR products can be isolated and sequenced to identify DNA fragments that encode
10 polypeptide sequences corresponding to the targeted region of human platelet F11 receptor.

Various modifications of the nucleic acid and amino acid sequences disclosed herein are covered by the subject invention. These varied sequences still encode a
15 functional human platelet F11 receptor. The invention thus further provides an isolated nucleic acid molecule encoding a human platelet F11 receptor, the nucleic acid molecule encoding a first amino acid sequence having at least 90% amino acid identity to a second amino acid
20 sequence, the second amino acid sequence having an amino acid sequence selected from the group consisting of SEQ ID NO:3, amino acid residues 28-299 of SEQ ID NO:3, SEQ ID NO:4, and amino acid residues 28-193 of SEQ ID NO:4. In further embodiments, the first amino acid sequence has
25 at least 95%, 96%, 97%, 98%, or 99% amino acid identity to SEQ ID NO:3, amino acid residues 28-299 of SEQ ID NO:3, SEQ ID NO:4, or amino acid residues 28-193 of SEQ ID NO:4.

The invention further provides an isolated DNA
30 oligomer capable of hybridizing to the nucleic acid molecule encoding the human platelet F11 receptor according to the subject invention. Such oligomers can be used as probes in a method of detecting the presence

of human platelet F11 receptor in a sample. More particularly, a sample can be contacted with the DNA oligomer and the DNA oligomer will hybridize to any human platelet F11 receptor present in the sample, forming a complex therewith. The complex can then be detected, thereby detecting presence of human platelet F11 receptor in the sample.

The complex can be detected using methods known in the art. Preferably, the DNA oligomer is labeled with a detectable marker so that detection of the marker after the DNA oligomer hybridizes to any human platelet F11 receptor in the sample (wherein non-hybridized DNA oligomer has been washed away) is detection of the complex. Detection of the complex indicates the presence of human platelet F11 receptor in the sample. As will be readily apparent to those skilled in the art, such a method could also be used quantitatively to assess the amount of human platelet F11 receptor in a sample.

For detection, the oligomers can be labeled with, for example, a radioactive isotope, biotin, an element opaque to X-rays, or a paramagnetic ion. Radioactive isotopes are commonly used and are well known to those skilled in the art. Representative examples include indium-111, technetium-99m, and iodine-123. Biotin is a standard label which would allow detection of the biotin labeled oligomer with avidin. Paramagnetic ions are also commonly used and include, for example, chelated metal ions of chromium (III), manganese (II), and iron (III). When using such labels, the labeled DNA oligomer can be imaged using methods known to those skilled in the art. Such imaging methods include, but are not limited to, X-ray, CAT scan, PET scan, NMRI, and fluoroscopy. Other suitable labels include enzymatic labels (horseradish

peroxidase, alkaline phosphatase, etc.) and fluorescent labels (such as FITC or rhodamine, etc.).

Fragments of the nucleic acid molecules encoding the F11 receptor are also provided, and are best defined in 5 the context of amino acid sequence relationships among members of the F11 receptor sequence family and information on the function of specific F11 receptor domains.

The F11 receptor molecule of the subject invention 10 can include a leader or signal sequence (for example, for targeting of the F11 receptor to the desired part of a cell). The leader can include an ATG start codon encoding a methionine residue. In fact, SEQ ID NOS:1-4 each include a signal sequence (amino acid residues 1-27 15 of SEQ ID NOS:3 and 4, encoded by nucleotides 16-96 of SEQ ID NOS:1 and 2, respectively). All versions of the F11 receptor (with and without 3' and 5' non-coding regions, and with and without the signal sequence) are thus specifically intended to be covered herein.

20 In some methods of the invention, tissues or cells are contacted with or exposed to a substance, including, for example, a composition or a compound. In the context of this invention, to "contact" tissues or cells with or to "expose" tissues or cells to a substance means to add 25 the substance, usually in a liquid carrier, to a cell suspension or tissue sample, either *in vitro* or *ex vivo*, or to administer the substance to cells or tissues within an animal (including humans).

Having thus detailed the various aspects of the 30 subject invention, the stimulatory anti-human platelet monoclonal antibody, termed M.Ab.F11 (IgG1 isotype) was identified and characterized previously as an agonist which induces platelet aggregation and granule secretion (Kornecki et al. 1990). The biochemical pathway by which

M.Ab.F11 induces platelet aggregation and secretion was shown to involve recognition by M.Ab.F11 of a platelet surface protein duplex termed the F11 receptor (Kornecki et al. 1990) (also called FAM, the F-11 Adhesion Molecule). This initial recognition of FAM was followed, in part, by crosslinking of the antibody through the Fc domain to the platelet Fc γ RII receptor (Naik et al. 1995). The binding of M.Ab.F11 to intact platelets resulted in the activation of signal transduction pathways leading to a transient, reversible translocation of PKC isozymes α and ζ from the cytoplasm to the membrane as well as a sustained, irreversible association of PKC isoenzymes δ , β , η' and θ at the plasma membrane (Wang et al. 1995). In addition, activation of platelets with M.Ab.F11 resulted in increased intracellular phosphorylation of p47, the protein kinase C substrate pleckstrin, and p20, the light chain fragment of myosin, which is also a substrate for myosin light chain kinase (Kornecki et al. 1990; Kornecki et al. 1995). The involvement of the fibrinogen receptor, the GPIIb/IIIa complex, in M.Ab.F11-induced platelet activation was shown by use of an anti-GPIIIa antibody which completely blocked the antibody-induced platelet aggregation and secretion (Kornecki et al. 1990).

FAM was initially identified immunologically as two membrane proteins of molecular weight of 32 and 35kDa (Kornecki et al. 1990). Following the purification of FAM by ion exchange and affinity chromatography, N-deglycosylation of either the 32 or 35kDa protein resulted in the formation of a deglycosylated core protein of 29kDa, recognized by M.Ab.F11 (Naik et al. 1995). Subsequently, twenty-six amino acids of the N-terminus of this protein were published (Naik et al. 1995) (SEQ ID NO:14: SVTVHSSEPEVRIPENNPKLSXAYS) (NCB

Accession # S56749), and several internal amino acid sequences, including forty-three amino acids of the endoproteinase (V8 protease) Glu-C proteolytic fragment (SEQ ID NO:15:WKFDQGDTTRLVEYNNKITASYEDRVTFLPTGIFTKSVTRED) 5 were detailed (Naik et al. 1995). Extensive data base searches performed at that time indicated that this molecule represented a new receptor on the platelet surface.

In accordance with the present invention, the 10 internal forty-three amino acid sequence of this molecule was utilized for the isolation of a cDNA encoding the full length FAM molecule. Sequence analysis demonstrated that FAM is an integral membrane protein and a novel member of the immunoglobulin superfamily. It was shown 15 to be present in platelets, endothelial cells and other cells of the hematopoietic system. Furthermore, autoantibodies developed against FAM were detected in the circulation of thrombocytopenic and renal disorder patients indicating that such antibodies may contribute 20 to the activation of platelets *in vivo*.

Experimental methods

Reagents. Electrophoresis-pure reagents, including acrylamide, ammonium persulfate, nitrocellulose, SDS, and 25 TEMED were purchased from BioRad (Hercules, CA). The 5' and 3' RACE systems were purchased from GIBCO BRL, the AdvanTAge PCR Cloning Kit from Clontech, the QIAprep Miniprep kit and the Rneasy Mini kit from Qiagen (Valencia, CA), Tag DNA polymerase and restriction 30 endonuclease Eco RI were purchased from Boehringer Mannheim (Indianapolis, IN). Fetal bovine serum was obtained from HyClone (Logan, UT). All other reagents were obtained from Sigma (St. Louis, MO) and were of the highest reagent grade.

Platelet isolation. Venous blood, obtained from healthy volunteers, was collected into the anticoagulant ACD (pH4.6) as previously described (Kornecki et al. 1990). The platelet-rich plasma (PRP) fraction was obtained by centrifugation (160 xg, 10 min.) and platelets were isolated in the presence of platelet aggregation inhibitors are described previously (Kornecki et al. 1990; Naik et al. 1995).

RNA isolation and processing. RNA was isolated in the following manner: isolated platelets were solubilized in "lysis buffer" (4M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% sodium sarcosine and 100 mM β -mercaptoethanol) dissolved in DEPC-treated water (Chomczynski and Sacchi 1987), passed 10-15X through a 21-gauge needle, diluted ten-fold with 2M sodium acetate (pH 4.0), and the RNA extracted by vigorous vortexing in the presence of an equal amount of the mixture containing 5 volumes of phenol (pH 4.0) and 1 volume of chloroform-isoamyl alcohol (29:1). The crude platelet RNA was precipitated with an equal volume of isopropanol. The precipitate was resolubilized in the "lysis buffer" and reprecipitated as above. For long-term storage, the RNA was kept as an isopropanol precipitate at -80°C. Immediately before RT-PCR amplification, the RNA was quantitated and its purity tested by measuring its absorbance at 260/280 nm. The quality of the isolated RNA was also tested by agarose gel electrophoresis.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) with degenerate primers. Reverse transcriptase reactions were carried-out using 1 μ g of the total RNA and the oligo d(T)16 primers using the Perkin Elmer kit as detailed in the manufacturer's protocol. The Polymerase Chain Reaction (PCR) was carried-out using degenerate primer pairs derived from the partial amino

acid sequence of the F11 receptor termed Glu-C (fragment 4), as previously described (Naik et al. 1995). The final concentration of each primer was 2 pmol/ μ l, while dNTP's and Mg²⁺ were present at 200 μ M and 2 mM, respectively. The PCR mix not containing the Taq polymerase was subjected to hot start at 99°C for 10 minutes followed by addition of the polymerase at 85°C to the final concentration of 1.25U per 50 μ l reaction. Thermocycling consisted of 5 cycles at 95°C for 1 min, 10 48°C for 2 min and 72°C for 2 min, followed by 45 cycles at 94°C for 1 min, 48°C for 2 min and 72°C for 2 min with a 1 sec extension per each subsequent cycle to compensate for the losses in Taq polymerase activity. The final extension was performed at 72°C for 5 min. Upon completion of PCR, samples were kept frozen and subjected to PAGE within several hours.

The names and sequences of the degenerate primers derived from the partial protein sequence of the F11 receptor were as follows: F11/f-6 (SEQ ID NO:16: 20 GARTAYAAYAAYAARATHAC), F11/r-5(A) (SEQ ID NO:17: TTRAANGTDATNCCNGTAGG), F11/r-5(C) (SEQ ID NO:18: TTRAANGTDATNCCNGTCGG), F11/r-5(G) (SEQ ID NO:19: TTRAANGTDATNCCNGTGGG), F11/r-5(T) (SEQ ID NO:20: TTRAANGTDATNCCNGTTGG). Primers used for 3' and 5'-RACE procedures were as follows: 25 GSP1 (SEQ ID NO:21: AGCTTCCTATGAGGACCGGG), GSP2 (SEQ ID NO:22: GTCACGGACTTGAAGGT), GSP4 (SEQ ID NO:23: GGCAAGAAGGTACCCGGTCC), GSP3 (SEQ ID NO:24: TTRAANGTDATNCCNGTTGG), Adapter Abridged Universal 30 Amplification Primers (AP and AUAP, GIBCO), 3'-F11-6F (SEQ ID NO:25: ATCTGGTTGCCTATAGCCG), 3'-F11-6R (SEQ ID NO:26: CTCTAGCCTCTAATTCCCGC).

DNA analysis by polyacrylamide gel electrophoresis (PAGE). The DNA samples (PCR products or plasmid DNA)

were separated by electrophoresis in 15% polyacrylamide slab minigels. Molecular weight standards of 50 bp DNA Ladder™, 100 bp DNA Ladder™ or 123 bp DNA Ladder™ (Gibco BRL, Gaithersburg, MD) were used to estimate the 5 approximate molecular weight of each DNA band. The gels were stained with ethidium bromide (5 μ g/ml), destained in distilled water and visualized using UV. Long wavelength UV (366nm) was used when the resolved DNA fragments were subsequently subjected to purification and cloning.

10 Purification and cloning of the amplified PCR products. Following PAGE, the DNA fragment was eluted by the standard "Crush and Soak Method" as described (Sambrook et al. 1989). The eluted DNA was digested with 1U of T4 DNA polymerase in the presence of 100 μ M dNTP, to 15 remove the protruding 3'-A overhangs, and the resulting blunt-ended product was phosphorylated at its 5' ends. The cloning vector, pBLUESCRIPT SK+ (Stratagene, La Jolla, CA) was linearized with SmaI and its 5'-ends dephosphorylated by two treatments with calf intestinal 20 alkaline phosphatase. Blunt end ligation was performed at 15°C for 18 hrs in the presence of T4 ligase. The molar ratio of insert to vector was approximately 5:1. The subsequent construct was transformed into DH5a competent cells (Gibco BRL, Gaithersburg, MD) as suggested by the 25 manufacturer. The plasmid DNA was isolated using the Wizard™ Midipreps DNA purification system (Promega, Madison, WI). The presence of the cloned insert was confirmed by KpnI and SacI digestion of the purified plasmid DNA. Nineteen colonies were selected as positive 30 and five of them have been sequenced.

DNA Sequencing. The isolated and purified plasmid DNA was sequenced by ACGT (Northbrook, IL). The fidelity of the data was confirmed by sequencing both strands using M13 universal forward and reverse primers.

3' Rapid Amplification of cDNA Ends (3'RACE). This procedure was used for the amplification of nucleic acid sequences from a mRNA template between the internal site of the Glu-C (fragment 4) determined above (SEQ ID NO:21: 5' AGCTTCCTATGAGGACCGGG 3', GSP1) and the 3' end of the mRNA. Total platelet RNA (0.5 µg) was prepared as described above. RT was carried-out using reverse transcriptase and the AP primer as described in the 3'RACE system (Gibco/BRL). The original mRNA template 10 was destroyed with RNase H. PCR was carried-out using the sequence-specific primer GSP1 and the Abridged Universal Amplification Primer (AUAP). Thirty-five cycles at 94°C for 5 sec, 58°C for 15 sec, and at 72°C for 3 min were performed. The final extension was performed at 15 72°C for 3 min. The PCR product was cloned into pT-Adv vector (Clontech, Palo Alto, CA) as described by the manufacturer. The plasmid DNA was subsequently purified using a QIAprep Miniprep Kit (QIAGEN, Valencia, CA) following procedures described in the QIAprep miniprep 20 handbook. Purified plasmid DNA was digested with EcoRI (Boehringer Manheim) following the manufacturer's protocol. The presence of the PCR product was confirmed using agarose gel electrophoresis (Sambrook et al. 1989). Six independent clones were sequenced by ACGT 25 (Northbrook, IL). One clone was sequenced in the forward and reverse direction using M13R and T7 universal primers. Four clones were sequenced on one strand, and one clone was sequenced on both directions using M13R, M13F and the sequence specific primers: GSP1, 3'-F11-6 30 forward primer (SEQ ID NO:25), and 3'-F11-6 reverse primer (SEQ ID NO:26). The GSP1 custom primer was synthesized by Ransom Hill Bioscience, and the 3'-F11-6 forward and 3'-F11-6 reverse primers were synthesized by Gibco/BRL.

5' Rapid Amplification of cDNA Ends (5'-RACE).

Total RNA was isolated from freshly-prepared human platelets (from 100 ml of whole blood) using a RNeasy Mini Kit (QIAGEN, Valencia, CA) following the procedures 5 described in the manufacturer's RNeasy Mini Handbook. The 5'-RACE system for Rapid Amplification of cDNA Ends (Version 2.0) (Gibco/BRL) was used to amplify the 5' end of the F11 cDNA following procedures detailed in the manufacturer's manual. The first strand cDNA synthesis 10 was primed using the gene-specific, antisense oligonucleotide (GSP2, SEQ ID NO:22: GTCACGGACTTGAAGGT). The product was purified from unincorporated dNTPs and GSP2 using a GlassMax DNA Isolation Spin Cartridge included in the kit. A homopolymeric tail was 15 subsequently added to the 3'-end of the cDNA using terminal deoxynucleotidyl transferase and dCTP. The dC-tailed cDNA was amplified by PCR using the gene-specific primer 3 (GCP3) (F11/r-5(RT), SEQ ID NO:27: TTRAAANGTDATNCCRGTTGG and the Abridged Universal Anchor 20 Primer (AUAP). Forty-five cycles at 94°C for 30 sec, 48°C for 1 min, and at 72°C for 3 min were performed, followed by a final extension at 72°C for 3 min. The diluted (1/100) primary PCR products were reamplified using a nested, gene specific primer 4 (GSP4, SEQ ID 25 NO:23: GGCAAGAAGGTACCCGGTCC) and Abridged Universal Amplification Primer (AUAP). Thirty-five cycles at 94°C for 30 sec, 55°C for 1 min, and at 72°C for 3 min were performed, followed by a final extension at 72°C for 3 min. The resulting PCR products were cloned into pT-Adv 30 vector (Clontech, Palo Alto, CA) and analyzed as described above for the 3'RACE system. Three clones were sequenced by ACGT (Northbrook, IL), two clones in both directions using M13R and M13F universal primers, and one

clone on one strand. All custom primers used for 5'-RACE procedure were synthesized by Gibco/BRL.

Data analysis. The translated amino acid sequence of FAM was analyzed using the following INTERNET based 5 software programs: TMpred, FASTA, ScanProsite, PfamHMM, PSORT, SSPRED, ProfileScan, ProtScale, PatScan, pI/Mw and Motif (available through Pedro's BioMolecular Research Tools at http://www.public.iastate.edu/~pedro/rt_1.html), SignalP(<http://www.cbs.dtu.dk/services/SignalP/output.html>), Pfam (The Sanger Centre, <http://www.sanger.ac.uk>) and Simple Modular Architecture Research Tool (<http://coot.embl-heidelberg.de/predictprotein>).

Cell preparation and cell culture. HEL cells were 15 purchased from the American Type Culture Collection. Megakaryocytic cell lines (CMK, CMK11-5 and CMK6) were obtained through the generous gift of Dr. H. Avraham, Hematology and Oncology Research Laboratory, New England Deaconess Hospital of Boston, MA. Cells were maintained 20 in RPMI 1640 medium supplemented with 10% FBS. Bovine aortic endothelial cells (BAEC) were obtained from Dr. George Tuszyński of the Medical School of Pennsylvania, PA. The human endothelial hybrid cell line (Eahy926) was obtained from Dr. Cora-Jean S. Edgell, University of 25 North Carolina, Chapel Hill, NC. The Eahy926 cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat inactivated FBS and HAT (100 μ mol hypoxanthine, 0.4 μ mol aminopterin, and 16 μ mol thymidine) in a humidified atmosphere containing 5%CO₂. The K562 cell 30 line was maintained in a 15% FBS-RPMI medium.

Electrophoresis and immunoblotting. Samples were prepared for SDS-PAGE as follows: for the preparation of RBC ghosts, the procedure of Schwoch and Passow was used (Schwoch and Passow 1973). Cell extracts of HEL, K562,

CMK, CMK6 and CMK11-5 cells were prepared as follows: cells were harvested, washed with PBS, and solubilized in Laemmli buffer. Eahy926, HUVEC, and BAEC cells were treated with 10mM EDTA-PBS, harvested, washed, suspended 5 in Laemmli buffer and processed for SDS-PAGE (Bio-Rad). Proteins were transferred onto nitrocellulose membranes and incubated for 16 hrs with the primary monoclonal (M.Ab.F11) or rabbit polyclonal FAM antibodies (4 µg/ml) or purified human antibodies, followed by incubation for 10 2 hrs with secondary antibodies, rabbit or goat anti-mouse/human Ig-AP conjugates (1:4000).

Flow cytometry. The CMK, CMK11-5, CMK6, HEL and K562 cell lines were washed with PBS and resuspended in 0.1% BSA/PBS buffer. HUVEC, Eahy926 and bovine endothelial 15 cells were treated with 10mM EDTA/PBS, washed with PBS and finally with 0.1% BSA/PBS. To isolate the buffy coat fraction, whole blood was mixed with equal amount of HBS buffer, overlaid on Ficoll and centrifuged. The buffy coat layer and erythrocytes were collected separately. 20 For flow cytometry analysis, the cells were resuspended in 0.1% BSA/PBS at the final concentration of 4×10^5 cells/sample and incubated with M.Ab.F11 (5µg/ml) or with an isotype identical nonreactive IgG (used as a control). Following a 1 hr incubation at 22°C, the cells were 25 washed with 0.1% BSA/PBS buffer, treated with 50µl of 1/100 diluted goat anti-mouse Ig-FITC (GAM), incubated for 30 min on ice, washed with 0.1% BSA/PBS and resuspended in 0.1% BSA/PBS. The samples were analyzed using a Becton Dickinson Immunocytometry Systems flow 30 cytometer (Becton Dickinson FAC Sort, San Jose, CA). Flow cytometric analysis of human platelets was performed in the presence of PGE₁.

Preparation of antibodies. M.Ab.F11 was identified and characterized as previously described (Kornecki et

al. 1990). A polyclonal antibody directed to the twenty-one amino acid N-terminal sequence of the F11 receptor (SEQ ID NO:5: SVTVHSSEPEVRIPENNPKLS, detailed in Naik et al. 1995) was prepared in rabbits by Qualify 5 Controlled Biochemicals, Inc (Hopkinton, MA) and subsequently purified by affinity chromatography.

Screening for FAM antibodies in patients. The presence of anti-FAM antibodies in the circulation of several patients was examined. Serum was obtained from a 10 64 year old male with a history of hypertension following two separate cerebrovascular accidents within a two-week period. While workup revealed no obvious source of the infarcts (carotid dopplers were normal and no thrombus was detected), the patient developed an aspiration 15 pneumonia and was treated with antibiotics. He developed acute renal failure and dialysis was initiated. At this time, his platelet count decreased from an admission level of 266,000/ml to 50,000/ml. The patient developed bilateral gangrene of both legs. Although the patient 20 had received heparin, it was only after the gangrene had developed, thus excluding a diagnosis of heparin-induced thrombocytopenia. The patient died three weeks after hospitalization due to overwhelming sepsis, respiratory failure and cardiac failure. Another patient examined 25 for the presence of FAM antibodies in his circulation was a 40 year old male who had end-stage renal disease secondary to hypertension. He had a cadaveric kidney transplant and was receiving the immune suppressive drug, tacrolimus (FK506).

30

EXAMPLE I

Immunological detection of FAM in platelet membranes.

It has been previously shown that monoclonal antibody F11 is able to detect two platelet membrane

glycoproteins of 32 and 35kDa, which have now been designated as FAM (Kornecki et al. 1990; Naik et al. 1995). For verification of the presence of FAM molecules as constituents of the platelet membrane, a polyclonal antibody was developed against the N-terminal portion of FAM, the twenty-one amino acid sequence which has previously been reported (Naik et al. 1995), and immunoblotting was used for the detection of these proteins. As shown in Fig. 1, immunoblotting of platelet proteins with the N-terminal, anti-FAM polyclonal antibody revealed the presence of both the 32/35kDa glycoproteins in the platelet membrane. These results were found to be identical to those obtained previously when the monoclonal antibody F11 was used for the detection of this duplex (Kornecki et al. 1990; Naik et al. 1995).

EXAMPLE II

Cloning of the F11R cDNA.

The amino acid sequence of the N-terminal portion of FAM molecule receptor and that of several peptides obtained following proteolytic digestion of the purified receptor were published previously (Naik et al. 1995). Based on the amino acid sequence of one of the fragments, Glu-C(fragment 4), containing SEQ ID NO:15:WKFDQGDTTRLVEYNNKITASYEDRVTFLPTGITFKSVTRED, four degenerate oligonucleotide primers (differing only by a single base, F 11/r-5(A), F11/r-5(C), F11/r-5(G), F11/r-5(T) and the forward primer F11/f-6 were designed and synthesized as described in the Experimental Methods section. Using platelet RNA and the designed primers, RT-PCR of the C-terminal portion of Glu-C(fragment 4) was carried-out. The PCR-amplified product was sequenced and translated. It was determined that the translated amino

acid sequence of the internal section of this product was identical to the internal amino acid sequence of the published Glu-C(fragment4) detailed above.

In order to obtain the full FAM cDNA sequence, 5 procedures were used which employed both the 5' and the 3' RACE techniques. The 3' RACE procedure used a 5' primer based on the nondegenerate sequence which was determined by PCR based on the reverse translation of the middle portion of Glu-C(fragment 4). The sequence of the 10 5' end of the 3'RACE clones was consistent with the predicted (degenerate) sequence obtained by reverse translation of the C-terminal and of the Glu-C (fragment 4) peptide.

5' RACE was carried-out using nested primers, the 15 sequences of which were directly derived from the 5' end of the clone obtained by the 3'RACE procedure. Analysis of the 5'RACE products revealed a reading frame containing the previously determined N-terminus of FAM (Naik et al. 1995), and the 26 N-terminal amino acids of 20 the Glu-C(fragment 4) peptide (Naik et al. 1995). The protein N-terminus was preceded by a signal sequence with a translation initiation codon having a consensus flanking sequence PuXXAUGG (Kozak 1987a; Kozak 1987b). The complete FAM cDNA and the deduced amino acid sequence 25 are shown in Fig. 2.

EXAMPLE III

Analysis of the FAM amino acid sequence.

Features of FAM apparent from analysis of the amino 30 acid sequence are summarized in Table 1. FAM expressed on human platelets is encoded by an mRNA of 1821 bases which upon translation yield a protein of 299 amino acids with a molecular weight of 29,682 kDa. The estimated value of the isoelectric point was 6.72 (Bjellqvist et

al. 1993; Bjellqvist et al. 1994). The mature protein (minus the signal sequence) has an amino acid sequence of 272 amino acids (amino acid residues 28-299 of SEQ ID NO:3).

5 Further data-base searches of potential membrane localization and orientation sites suggested that FAM is an integral membrane protein with a potential transmembrane domain (amino acids 239 to 258 of SEQ ID NO:3), a cytoplasmic-oriented C-terminus (amino acids 255 10 to 299 of SEQ ID NO:3), and an extracellular N-terminus (amino acids 28 to 239 of SEQ ID NO:3), and contains a cleavable signal sequence corresponding to amino acids 1 to 27 of SEQ ID NO:3 (Nielsen et al. 1997; Singer 1990). Also, these analyses indicated that FAM contains one 15 potential N-glycosylation site (Bause 1983; Gavel and von Heijne 1990; Miletich and Broze 1990). The cytoplasmic tail of the FAM was shown to contain two phosphorylable tyrosine residues at positions 260 and 279 of SEQ ID NO:3 as well as putative intracellular and extracellular sites 20 of phosphorylation by casein kinase II (Pinna 1990), protein kinase C (Woodget et al. 1986; Kishimoto et al. 1985) and cAMP- or cGMP-dependent protein kinases (Glass and Smith 1983; Glass et al. 1986). Hydrophobicity analysis of the FAM amino acid sequence, performed using 25 the ProtScale program following the method of Kyte and Doolittle (Kyte and Doolittle 1982), is shown in Fig 3.

EXAMPLE IV

FAM is a novel Ig superfamily member.

30 A combined literature and protein database search of sequences homologous to FAM revealed that FAM exhibits two regions (amino acids 41 to 116 and 144 to 219 of SEQ ID NO:3; SEQ ID NOS:9 and 11, respectively) of high homology to members of the immunoglobulin superfamily

(IgSF) (Kyte and Doolittle 1982; Williams and Barclay 1988; Williams et al. 1989; Bork et al. 1994; Brummendorf and Rathjen 1995; Karplus et al. 1997; Schultz et al. 1998). These two amino acid sequences, representing 5 putative Ig domains, were compared to the conserved patterns for Ig folds (SEQ ID NOS:8 and 10) using the SMART program, and based on their length, sequence similarity and secondary structure analysis, FAM was found to be comprised of two C2-type immunoglobulin 10 domains (amino acids 41 to 116 and 144 to 219 of SEQ ID NO:3; SEQ ID NOS:9 and 11, respectively), as shown in Figs. 4A and 4B. Localization of the C2 Ig domains within the structure of FAM is illustrated in the schematic model shown in Fig. 5.

15

EXAMPLE V

Search for homologous cDNA and protein sequences.

Comparisons of the FAM cDNA sequence against all known sequences in the GenBank Database and a literature 20 search revealed homology of FAM to the recently cloned murine protein, junctional adhesion molecule (JAM) and to an antigen recognized by the A33 monoclonal antibody (Fig. 6). FAM appears to be a human homolog of murine JAM, which was shown to be localized at intracellular 25 junctions of mouse endothelial and epithelial cells (Martin-Padura et al. 1998). The A33 antigen is a transmembrane glycoprotein expressed in gastrointestinal epithelium and in 95% of human colon cancers (Welt et al. 1990; Heath et al. 1997; Moritz et al. 1998). As shown 30 in Fig. 6, a comparison of the amino acid sequences of the FAM (SEQ ID NO:3), JAM (SEQ ID NO:12) and A33 (SEQ ID NO:13) indicates that they contain a single transmembrane domain and two Ig folds. However, the two Ig domains of FAM are both of the C-2 type, whereas the first Ig domain

of the A33 and JAM proteins have been categorized as V-type by the authors (Martin-Padura et al. 1998; Heath et al. 1997; Moritz et al. 1998).

5

EXAMPLE VI

Cellular distribution of the FAM molecule.

Fluorescence flow cytometry and immunoblotting procedures using M.Ab.F11 were used to examine the expression of FAM in various hematopoietic cells and cell lines. As shown in Figs. 7A-7H, significant FAM was detected in parent CMK cells and subclones CMK6 and CMK11-5, HEL, K562, HUVEC, and the human endothelial hybrid cell line, Eahy 926, and erythrocytes and lymphocytes. Consistent with these observations was the identification of several glycosylated forms of FAM by immunoblotting. As shown in Fig. 7I, FAM was detected as a 32/35 kDa duplex in platelets, a single protein band of 35 kDa in red cells, and a broad protein band of 33/36kDa in HUVEC and bovine aortic endothelial, K562 cells and human lymphocytes (data not shown).

EXAMPLE VII

Identification of FAM antibodies in the circulation of patients.

Fig. 8 demonstrates the presence of FAM antibodies in the circulation of a thrombocytopenic patient (lane 3) and a renal disorder patient (lane 4). As shown in Lane 1, the murine monoclonal M.Ab.F11 detected both of the 32/35kDa proteins in control platelet proteins. In comparison to M.Ab.F11, it appears that only the 32kDa form of FAM could be detected by antibodies obtained from a thrombocytopenic patient (lane 3). Antibodies obtained from a renal disorder patient appear to detect both the 35kDa as well as the 32kDa forms (lane 4). In contrast,

the plasmas of normal, healthy donors did not contain FAM antibodies, evidenced by a complete lack of FAM-labeling by normal plasma in lanes 5-7.

5 Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the
10 invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

Table 1

5 Properties of the FAM obtained by analysis
of its amino acid composition deduced
from cDNA sequence.

Properties		Amino Acids	
10	Molecular Weight	29,682	kDa
	Isoelectric point	6.72	
	Transmembrane helices	12-29	inside→outside
		239-258	outside→inside
15	The cleavable N-term signal sequence	1-27	
	The cytoplasmic tail	255-299	
	N-glycosylation site	Asn-185	
	cAMP-/or cGMP-dependent protein kinase phosphorylation site	Thr-273	
	PKC phosphorylation sites	Ser-57 Thr-69 Thr-95 Ser-179	Thr-269 Ser-274 Ser-275 Ser-284
20	Casein kinase II phosphorylation sites	Ser-34 Ser-82 Thr-100 Ser-118 Thr-152	Ser-154 Thr-193 Ser-203 Ser-287
	Tyrosines in the cytopl. tail	Tyr-260	Tyr-279

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